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(54) Title: AN ENZYME PREPARATION FOR IMPROVED BAKING QUALITY AND A PROCESS FOR PREPARING THE SAME

(57) Abstract: The present invention provides an improved method for the preparation of an enzyme mixture for improved freshkeeping of baked products. The enzyme preparation is produced by the co culture of two fungal species of *Rhizopus & Aspergillus* by solid substrate fermentation under optimal fermentation parameters. The product is extracted and further processed to get an enzyme preparation as solid powder. The present invention also includes an enzyme preparation obtained from obtained from *Rhizopus sp.* and*Aspergillus sp.*.

AN ENZYME PREPARATION FOR IMPROVED BAKING QUALITY AND A PROCESS FOR PREPARING THE SAME

The present invention relates to an enzyme preparation for improved baking quality and a process for preparing the same

BACKGROUND

The process of baking has been in existence for at least 6000 years and has been exploited ever since to improve the process. The major components of baked products are polysaccharides ,proteins, fats & emulsifiers, enzymes, yeast and water. Of these enzymes are involved in the oxidative aging of flour, in dough development and in the formation of bread flavor & texture. They are also responsible for a number of adverse effects on the quality of the baked product. On the whole the overall effect of the indigenous enzymes of the grain, the microorganisms & of various added exogenous enzymes influence the quality of the baked product (Chemistry and physics of baking. Ed. J.M.V. Blanshard, P. J. Frazier & T. Galliard, Published by The Royal Society of Chemistry 1986).

Staling of baked products is a major negative influence & is defined as an increase of crumb firmness & a corresponding loss of moisture & freshness. During staling there is a gradual transition of the polysaccharide starch from an amorphous to a partially crystalline state. This increase in crystallinity called retrogradation is due to an inter or intra molecular association of starch molecules via hydrogen bonding . The staling process is influenced by time, temperature, moisture & presence of additives in the baked product (Enzymes used in the milling industry. Ter Haseborg .E Alimenta,1988,27,1,2-10).

Additives act by way of complexing with the components of starch - amylose & amylopectin , thereby reducing their tendency to retrograde. Increasingly , enzymes too are being added to prevent staling. These antistaling enzymes are generally alpha & beta amylases, glucoamylases, glucose oxidase, peroxidases and lipoxygenases.

For a successful antistaling enzyme it is essential that they can act on raw starch rather than gelatinised starch & have an intermediate heat stability i.e. more stable than the conventional heat labile fungal amylases but less stable than the conventional heat stable bacterial amylases. These enzymes would thus provide an antistaling effect without adversely affecting the quality of the baked product , whereby they hydrolyse starch during the dough development process, but are completely heat inactivated before the baking process is completed. They thus prevent an excess of the breakdown products of starch - the dextrins which lead to an increased stickiness & gumminess of the bread (Enzymes for the baking industry . Van Oort,M.G, Hamer, R.J Aliment.,Equipos Tecnol,1993, 12,5,115 –18). The antistaling enzyme should, besides aiding fresh keeping of bread should not adversely affect the other desirable parameters of a baked product like loaf volume, color, softness, resilience & crumb texture (Synergistic effect of enzymes for breadbaking . Si,J.Qi Cereal Foods World 9, 1997, 42,10,802-807).

Co culture of different strains of bacteria - bacteria (Deanda et. al., Appl Environ Microbiol, 1996,62,12,4465-70), yeast –bacteria (Kim et. al., Biotechnol Lett, 1996,18,9,1031-34), yeast –yeast , yeast / bacteria – fungi (Padmaja et. al. J. Sci Food Agric, 1993, **63**,4,473-81; Marakis S.G Biotechnol Lett, 1992,14,11,1075-80) & fungi –fungi (Benzuela Elegado, Francisco, Fujio,Yusaku J. Gen. Appl.

Microbiol.1993,39,6, 541-6; Gutierrez-Correa, Marcel; Tengerdy,Robert P , Biotechnol. Lett. 1997,19,7,665-667; Cellulase production by mixed fungi in solid substrate fermentation of bagasse (Duenas R et. Al. World T. Microbiol. Biotechnol ,1995 ,11,3,333-337) have been reported in literature. . These associations have been used to produce many specialty products like acetic acid, alcohol butanol & enzymes like xylanase (Xylanase production by fungal mixed culture solid substrate fermentation on sugarcane bagasse. Gutierrez-Correa, Marcel; Tengerdy,Robert P , Biotechnol. Lett. 1998,20,1,45 – 47) and cellulases (Denada et. al., Appl Environ Microbiol, 1996,62 12,4465-70). The latter has been obtained using Trichoderma & Aspergillus strains (Mixed culture solid substrate fermentation of Trichoderma reesei with Aspergillus niger on sugarcane bagasse. Gutierrez-Correa, Marcel; Portal Leticia, Moreno Patricia, Tengerdy, Robert P, Bioresour. Technol , 1998, Volume date 1999, 68,2,173-178; Production of cellulase on sugarcane bagasse by fungal mixed culture solid substrate fermentation. Gutierrez-Correa, Marcel; Tengerdy,Robert P , Biotechnol. Lett. 1997,19,7,665-667; Formation of cellulases by co – culturing of Trichoderma reesei and Aspergillus niger on cellulosic waste. Madamwar,D ,Patel,S, World J. Microbiol.Biotechnol 1992, 8,2,183-6).

DESCRIPTION OF THE INVENTION

The present invention relates to a novel process to produce an enzyme preparation with antistaling properties for improved freshkeeping of baked products.

Accordingly, the present invention provides for a A method for the manufacture of an enzyme preparation for improved baked products, which comprises of:

a. preparing an inoculum of *Rhizopus sp.*,

b. preparing an inoculum of *Aspergillus sp.*,
c. mixing the grown inoculum of *Rhizopus sp.* and *Aspergillus sp.*,
d. sterilizing a solid state nutritive matrix,
e. mixing the said sterilized solid state nutritive matrix with the mixture of inoculum consisting of *Rhizopus sp.* and *Aspergillus sp.*,
f. incubating the said inoculated solid state nutritive matrix for 4 - 7 days at 25 - 35°C,
g. extracting the fermented matrix followed by filtration,
h. concentrating the aqueous extract,
i. spray drying of the concentrated aqueous extract to get the **enzyme preparation**.

The *Rhizopus sp.* is *Rhizopus oryzae.* The *Aspergillus sp.* is *Aspergillus niger.* The inoculum of *Rhizopus sp.* and *Aspergillus sp.* is mixed in the ratio ranging from 3:97 to 97:3. The inoculum of *Rhizopus sp.* and *Aspergillus sp.* is mixed in the ratio of 12.5 : 87.5. The solid state nutritive matrix is selected from wheat bran, rice bran, soya grits, rice grits, millet flour, soya flour, sugar beet, bagasse or a mixture of these. The mixed inoculum is optionally grown prior to mixing with sterilized solid state matrix. The extraction is carried out using water or an aqueous buffer. The aqueous extract is concentrated by ultra-filtration or reverse osmosis. In step (e) at least 10 % of inoculum is mixed with the solid state matrix. In step (f), the inoculate solid state matrix is incubated at 30 °C. The quantity of water added to the extract is in the ration of 1:6. The spray dried enzyme preparation is granulated. The spray dried enzyme preparation contains a preservative. The preservative is selected from benzoates or sorbates. The spray dried enzyme preparation contains a stabilizer. The stabilizer is selected from inorganic salts, polyols, sugars or their combinations. The spray drying step in 1 (i) is replaced by freeze drying or solvent precipitation. The inoculum is optionally not mixed but added together to the sterilized nutritive matrix.

The enzyme preparation is used for baking for improved freshkeeping, whitening, softening, crumb texture or volume increase.

The present invention also comprises an enzyme preparation for improving baked products obtained from *Rhizopus sp.* and *Aspergillus sp.* The *Rhizopus sp.* is *Rhizopus oryzae.* The *Aspergillus sp.* is *Aspergillus niger.* The enzyme preparation further comprises a preservative, preferably selected from benzoates or sorbates. The enzyme preparation further comprises a stabilizer, preferably selected from inorganic salts, polyols, sugars or their combinations .

The present invention has the following advantages over the other reported methods:

(i) The enzyme preparation obtained is from a non-genetically modified (GMO) origin.

(ii) The process is environment friendly, less cumbersome and economical for large-scale industrial applications.

(iii) A broader range of enzyme activities that aid freshkeeping is obtained , which is not obtained with a single culture alone.

(iv) This combination of desired activities for freshkeeping is generated *in situ* thereby having an advantage over blended enzyme preparations.

(v) The enzyme preparation is capable of acting on raw starch , rather than gelatinized starch.

The invention will now be described with reference to the following examples:

EXAMPLE 1

Preparation of inoculum of *Rhizopus sp*:

About 100 µL spore suspension of *Rhizopus oryzae*, made by adding 3 mL of sterile water to a culture slant, is added to a 250 mL Erlenmeyer flask containing 35 mL of medium with following composition:

No	Ingredient	Quantity (%)
1	Wheat flour	4.14
2	Sucrose	1
3	Peptone	0.306
4	Ammonium sulphate	0.2
5	Yeast extract	0.2
6	Potassium dihydrogen phosphate	0.085
7	$CaCl_2.2H_2O$	0.01
8	$MgSO_4.7H_2O$	0.01
9	NaCl	0.01

The flasks are incubated at 30 deg C on a rotary shaker (200-rpm) for 48 hours.

EXAMPLE 2

Preparation of inoculum of *Aspergillus sp*:

About 100 µL spore suspension of *Aspergillus niger*, made by adding 3 mL of sterile water to a culture slant, is added to a 250 mL Erlenmeyer flask containing 35 mL of medium with following composition:

No	Ingredient	Quantity (%)
1	Wheat flour	4.14

2	Peptone	0.306
3	Mono ammonium phosphate	0.0162
4	Amycoglucosidase	0.0054

The flasks are incubated at 30 deg C on a rotary shaker (200-rpm) for 48 hours.

EXAMPLE 3

Mixing of the inoculum

The inoculum developed from Example 1 is mixed the inoculum of Example 2 in the ratio 12.5:87.5. This mixed inoculum is used in the subsequent stages.

Example 4

Sterilization of wheat bran:

Commercial wheat bran is used as the solid substrate. Moist wheat bran is autoclaved for 90minutes in a rotating cooker.

Example 5

Inoculation:

The mixed inoculum from example 3 is mixed with the sterilized wheat bran from example 4. A 10 % inoculum is used. The inoculum is mixed at 30 deg. C. After a proper mixing, the inoculated wheat bran is transferred and layered into the pre-sterilized trays.

Example 6

Incubation:

Incubation of the layered trays from example 5 is done at 30 deg.C. for 5 days. At the end of 5 days, the fermented solid substrate is harvested.

Example 7

Extraction:

Extraction is done using water. Quantity of water added to the extract is in a ratio of 1:6. Soaking of the harvested solid substrate is done for 6 hours.

Example 8

Extraction:

The extraction is carried out in the same way as example 7 but with the addition of 0.2% sodium benzoate with the water.

Example 9

Microfiltration:

Microfiltartion is done to the extract obtained from example 7 to obtain a microorganism free product. This is done using a 0.2 micron MF membrane.

Example 10

Ultrafiltration:

The resultant liquid obtained from example 9 is concentrated to the desired activity by Ultrafiltration done at 15 deg.C. Ultrafiltration is done using UF membranes.

Example 11

Enzyme stabilization:

The Ultrafiltered enzyme obtained from example 10 is stabilized by the addition of NaCl and KCl.

Example 12

Enzyme stabilization:

The Ultrafiltered enzyme obtained from example 10 is stabilized by the addition of gylcerol and sorbitol.

Example 13

Spray drying:

The UFC (ultrafiltered concentrate) as obtained from example 10 is spray dried to get the required enzyme preparation.

Example 14

Spray drying:

The UFC (ultrafiltered concentrate) as obtained from Example 11 is spray dried as in Example 13, but with the addition of 0.1 % potassium sorbate as a preservative before spray drying.

Example 15

Freeze drying:

The UFC (ultrafiltered concentrate) as obtained from Example 11 is freeze dried.

Example 16

Baking trials:

The enzyme preparation obtained from example 15 was used in a baking trial. This freeze dried enzyme was dosed at 113 ppm in a bread baking trial & was evaluated for fresh keeping against a trial which did not have the enzyme.

The results of the baking trials of the test sample (containing the enzyme preparation), were compared with the control (where the enzyme preparation was absent) and the quality of the bread evaluated. The following observations were made:

DAYS	**EVALUATION**	**CONTROL**	**TEST**
	Enzyme preparation	Absent	Present
05	Softness	+	+++
	Mold Growth	-	-
	Slice strength	Good	Good
10	Softness	+	+++
	Mold Growth	+	-
	Slice strength	Weak	Weak
14	Softness	+	+++
	Mold Growth	++	+
	Slice strength	Weak	Weak
	OVERALL RATING	+	+++

The bread made with the freeze dried enzyme preparation appeared softer than that of the control even after 14 days.

Note: + denotes poor
++ denotes average
+++ denotes good
- denotes absent

We claim:

1. A method for the manufacture of an enzyme preparation for improved baked products, which comprises of:
   j. preparing an inoculum of *Rhizopus sp.*,
   k. preparing an inoculum of *Aspergillus sp.*,
   l. mixing the grown inoculum of *Rhizopus sp.* and *Aspergillus sp.*,
   m. sterilizing a solid state nutritive matrix,
   n. mixing the said sterilized solid state nutritive matrix with the mixture of inoculum consisting of *Rhizopus sp.* and *Aspergillus sp.*,
   o. incubating the said inoculated solid state nutritive matrix for 4 - 7 days at 25 - 35°C,
   p. extracting the fermented matrix followed by filtration,
   q. concentrating the aqueous extract,
   r. spray drying of the concentrated aqueous extract to get the **enzyme preparation**.
2. The method as claimed in claim 1 wherein the *Rhizopus sp.* is *Rhizopus oryzae.*
3. The method as claimed in claim 1 wherein the *Aspergillus sp.* is *Aspergillus niger.*
4. The method as claimed in claim 1 wherein the inoculum of *Rhizopus sp.* and *Aspergillus sp.* is mixed in the ratio ranging from 3:97 to 97:3.
5. The method as claimed in claim 4 wherein the inoculum of *Rhizopus sp.* and *Aspergillus sp.* is mixed in the ratio of 12.5 : 87.5.

6. The method as claimed in claim 1 wherein the solid state nutritive matrix is selected from wheat bran, rice bran, soya grits, rice grits, millet flour, soya flour, sugar beet, bagasse or a mixture of these.

7. The method as claimed in claim 1 wherein the mixed inoculum is optionally grown prior to mixing with sterilized solid state matrix.

8. The method as claimed in claim 1 wherein the extraction is carried out using water or an aqueous buffer.

9. The method as claimed in claim 1 wherein the aqueous extract is concentrated by ultra-filtration or reverse osmosis.

10. The method as claimed in claim 1 wherein in step (e) at least 10 % of inoculum is mixed with the solid state matrix.

11. The method as claimed in claim 1 wherein in step (f), the inoculate solid state matrix is incubated at 30 °C.

12. The method as claimed in claim 8 wherein quantity of water added to the extract is in the ration of 1:6.

13. The method as claimed in claim 1 wherein the spray dried enzyme preparation is granulated.

14. The method as claimed in claim 1 or 13 wherein the spray dried enzyme preparation contains a preservative.

15. The method as claimed in claim 14 wherein the preservative is selected from benzoates or sorbates.

16. The method as claimed in any one of the preceding claims wherein the spray dried enzyme preparation contains a stabilizer.

17. The method as claimed in claim 16 wherein the stabilizer is selected from inorganic salts, polyols, sugars or their combinations .

18. The method as claimed in claim 1 to 17 wherein the spray drying step in 1 (i) is replaced by freeze drying or solvent precipitation.

19. The method as claimed in claim 1 to 14 wherein the inoculum is optionally not mixed but added together to the sterilized nutritive matrix.

20. The method as claimed in claim 1 to 14 wherein the enzyme preparation is used for baking for improved freshkeeping, whitening, softening, crumb texture or volume increase.

21. An enzyme preparation for improving baked products obtained from *Rhizopus sp.* and *Aspergillus sp.*

22. An enzyme preparation as claimed in claim 21 wherein *Rhizopus sp.* is *Rhizopus oryzae.*

23. An enzyme preparation as claimed in claim 22 wherein the *Aspergillus sp.* is *Aspergillus niger.*

24. An enzyme preparation as claimed in claim 21 wherein the enzyme preparation is granulated.

25. An enzyme preparation as claimed in any one of claims 21- 24 further comprising a preservative.

26. An enzyme preparation as claimed in claim 25 wherein the preservative is selected from benzoates or sorbates.

27. An enzyme preparation as claimed in any one of the preceding claims further comprising a stabilizer.

28. An enzyme preparation as claimed in claim 27 wherein the stabilizer is selected from inorganic salts, polyols, sugars or their combinations .

AMENDED CLAIMS

[received by the International Bureau on 15 March 2002 (15.03.02);
original claims 1-28 replaced by new claims 1-20 (3 pages)]

1. A method for the manufacture of an enzyme preparation for improved baked products, which comprises of:
   a. preparing an inoculum of *Rhizopus sp.*,
   b. preparing an inoculum of *Aspergillus sp.*,
   c. mixing the grown inoculum of *Rhizopus sp.* and *Aspergillus sp.*,
   d. sterilizing a solid state nutritive matrix,
   e. mixing the said sterilized solid state nutritive matrix with the mixture of inoculum consisting of *Rhizopus sp.* and *Aspergillus sp.*,
   f. incubating the said inoculated solid state nutritive matrix for 4 - 7 days at 25 - 35°C,
   g. extracting the fermented matrix followed by filtration,
   h. concentrating the aqueous extract,
   i. spray drying of the concentrated aqueous extract to get the enzyme preparation.

2. The method as claimed in claim 1 wherein the *Rhizopus sp.* is *Rhizopus oryzae.*

3. The method as claimed in claim 1 wherein the *Aspergillus sp.* is *Aspergillus niger.*

4. The method as claimed in claim 1 wherein the inoculum of *Rhizopus sp.* and *Aspergillus sp.* is mixed in the ratio ranging from 3:97 to 97:3.

5. The method as claimed in claim 4 wherein the inoculum of *Rhizopus sp.* and *Aspergillus sp.* is mixed in the ratio of 12.5 : 87.5.

6. The method as claimed in claim 1 wherein the solid state nutritive matrix is selected from wheat bran, rice bran, soya grits, rice grits, millet flour, soya flour, sugar beet, bagasse or a mixture of these.

7. The method as claimed in claim 1 wherein the mixed inoculum is optionally grown prior to mixing with sterilized solid state matrix.

8. The method as claimed in claim 1 wherein the extraction is carried out using water or an aqueous buffer.

9. The method as claimed in claim 1 wherein the aqueous extract is concentrated by ultra-filtration or reverse osmosis.

10. The method as claimed in claim 1 wherein in step (e) at least 10 % of inoculum is mixed with the solid state matrix.

11. The method as claimed in claim 1 wherein in step (f), the inoculate solid state matrix is incubated at 30 °C.

12. The method as claimed in claim 8 wherein quantity of water added to the extract is in the ration of 1:6.

13. The method as claimed in claim 1 wherein the spray dried enzyme preparation is granulated.

14. The method as claimed in claim 1 or 13 wherein the spray dried enzyme preparation contains a preservative.

15. The method as claimed in claim 14 wherein the preservative is selected from benzoates or sorbates.

16. The method as claimed in any one of the preceding claims wherein the spray dried enzyme preparation contains a stabilizer.

17. The method as claimed in claim 16 wherein the stabilizer is selected from inorganic salts, polyols, sugars or their combinations .

18. The method as claimed in claim 1 to 17 wherein the spray drying step in 1 (i) is replaced by freeze drying or solvent precipitation.

19. The method as claimed in claim 1 to 14 wherein the inoculum is optionally not mixed but added together to the sterilized nutritive matrix.

20. The method as claimed in claim 1 to 14 wherein the enzyme preparation is used for baking for improved fresh keeping, whitening, softening, crumb texture or volume increase.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/00 C12N9/98 C12N1/14 C12P21/00 A21D8/04
//(C12N9/00,C12R1:685),(C12N9/00,C12R1:845),(C12N9/98,C12R1:685),
(C12N9/98,C12R1:845),(C12P21/00,C12R1:685),(C12P21/00,C12R1:845)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P C12R C12M A21D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, FSTA, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIM H-S ET AL.: "Enzymological characteristics and identification of useful fungi isolated from traditional Korean Nuruk." KOREAN JOURNAL OF APPLIED MICROBIOLOGY AND BIOTECHNOLOGY , vol. 26, no. 5, 1998, pages 456-464, XP001051554 abstract table 8 --- -/--	1,2

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
7 January 2002	23/01/2002

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Inte nal Application No
PCT/IN 01/00094

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 198621 Derwent Publications Ltd., London, GB; Class D16, AN 1986-134353 XP002185477 & JP 61 070980 A (NIIGATA PREFECTURE), 11 April 1986 (1986-04-11) abstract	1,21
X Y	EP 0 585 988 A (GIST BROCADES NV) 9 March 1994 (1994-03-09) page 2, line 47 -page 3, line 7 example 1 claims 1-4	21,22, 25-28 24
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BIGELIS R: "Handbook of applied mycology, Volume 3: Foods and feeds (eds Arora D K et al.), Chapter 14: FUNGAL ENZYMES IN FOOD PROCESSING (pp 445-498)" 1991 , MARCEL DEKKER, INC. , NEW YORK, USA; ISBN 0-8247-8491-X. XP001051671 page 445, line 15 -page 449, line 8 page 476, line 1 -page 478, line 9	21
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